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# Formation and mitigation of *N*-nitrosamines in nitrite preserved cooked sausages



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*N*-nitrosoproline (NPRO) (PubChem CID: 24141)

*N*-nitrosohydroxyproline (NHPRO) (PubChem CID: 61873)

*N*-nitrosopiperidine (NPIP) (PubChem CID: 7526)

*N*-nitrososarcosine (NSAR) (PubChem CID: 25811)

*N*-nitrosodimethylamine (NDMA) (PubChem CID: 6124)

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## ABSTRACT

Literature on formation and mitigation of *N*-nitrosamine (NA) and especially non-volatile NA (NVNA) in meat products is scarce and the present study is therefore a relevant contribution to the field. We found positive correlation between the levels of *N*-nitrosopiperidine (NPIP), *N*-nitrosohydroxyproline (NHPRO), *N*-nitrosoproline (NPRO), *N*-nitrosothiazolidine-4-carboxylic acid (NTCA) and *N*-nitroso-2-methyl-thiazolidine-4-carboxylic acid (NMTCA) and the amount of nitrite added to cooked pork sausages. The levels studied were 0, 60, 100, 150, 250 and 350 mg kg<sup>-1</sup>. The levels of *N*-nitrosodimethylamine (NDMA) and *N*-nitrosopyrrolidine (NPYR) remained at or below limit of quantification. Erythorbic acid inhibited the formation of NHPRO, NPRO, NPIP and NTCA. This inhibition was for NTCA and NMTCA counteracted by addition of free iron. Ascorbyl palmitate had less inhibitory effect than erythorbic acid and a combination of the two provided no further protection. Increasing the black pepper content increased the levels of NPIP and NMTCA. Only slight effects of increased fat content and addition of tripolyphosphate were observed.

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## 1. Introduction

Sodium nitrite (nitrite) has for decades been widely used for preservation of meat products and is an efficient inhibitor of the growth of *Clostridium botulinum* and thereby decreases the risk of this organism producing toxins and heat-resistant spores. Nitrite also provides the processed meat with its characteristic red colour,

flavours and aromas, known from products such as bacon, and it inhibits lipid oxidation processes (Skibsted, 2011). However, *N*-nitrosamines (NA) may be formed during production and storage of nitrite preserved meat products. The group of NA include both the so called volatile NA (VNA) and the non-volatile NA (NVNA). The levels of these compounds in nitrite preserved meat products varies greatly, from below detectability (<1 µg kg<sup>-1</sup>) to levels in the order of thousands µg kg<sup>-1</sup>, depending on the type of NA. In particular the NVNA are found in high amounts (Hill et al., 1988). The NA is a large group of compounds of which the

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majority is carcinogenic (IARC, 1978). The VNA are generally potent carcinogens (e.g. *N*-nitrosodimethylamine (NDMA) and *N*-nitrosopyrrolidine (NPYR)) whereas the NVNA are weak carcinogens (*N*-nitrososarcosine (NSAR)), or assumed to be non-carcinogenic (e.g. *N*-nitrosoproline (NPRO), *N*-nitrosohydroxyproline (NHPRO), *N*-nitroso-thiazolidine-4-carboxylic acid (NTCA) and *N*-nitroso-2-methyl-thiazolidine 4-carboxylic acid (NMTCA)). However, the assumption that NVNA as NPRO, NHPRO, NTCA and NMTCA are non-carcinogenic, needs to be verified by actual toxicological *in vivo* studies. Theoretically there is a risk of these compounds being decarboxylated into their carcinogenic counterparts (NPYR, NHPYR, NthZ and NMThZ) either during heat treatment or by microbial activity in the large intestine. As long as it cannot be verified whether these presumable non-carcinogenic NA, contribute to the adverse health effects observed by consumption of processed meat or not, their formation should be prevented as much as possible.

Studies have indicated that there is a positive though not necessarily linear correlation, between the amount of nitrite added and the amount of NA formed (Drabik-Markiewicz et al., 2009; Drabik-Markiewicz et al., 2011; Yurchenko & Mölder, 2007). These studies also indicate that the effects observed on the NA levels by changes in the amount of nitrite added during preparation, i.e. the ingoing amount of nitrite, may be different for the different NA and/or for the different test systems/meat products. Furthermore, the majority of the available publications only deal with the VNA, i.e. typically NDMA, *N*-nitrosodiethylamine (NDEA), NPYR and *N*-nitrosopiperidine (NPIP). Thus, data on the possible relationship between ingoing amount of nitrite and the extent of NA formation in a meat product for both VNA and NVNA are scarce or non-existing.

Besides the ingoing amount of nitrite a wide range of factors may potentially affect the formation of NA. These factors are related to meat quality, fat content, processing, maturation and handling at home. Factors related to processing include additives, heat applied during drying or smoking, precursors (added via wood smoke, spices or other ingredients), storage/maturation conditions and packaging. Processing factors can easily be controlled and their role in NA formation have been widely studied (Hill et al., 1988; Li, Wang, Xu, & Zhou, 2012; Li, Shao, Zhu, Zhou, & Xu, 2013; Sebranek & Fox, 1985). These studies only deal with the VNA (NDMA, NPYR and in a few cases NDEA), whereas studies including the NVNA are scarce (Janowski, Eisenbrand, & Preussmann, 1978).

Antioxidants are widely used as additives in meat processing because they increase the storage stability. There is a large amount of literature on the effects of antioxidants on lipid oxidation processes, whereas literature on the effect on the NA formation in meat products is limited (Li et al., 2012; Li et al., 2013; Mottram, Patterson, Rhodes, & Gough, 1975; Rywotycki & Ryszard, 2002; Sen, Donaldson, Seaman, Iyengar, & Miles, 1976). These studies on the effect of adding antioxidants to meat also only deal with NDMA, NPYR and NDEA and to our knowledge only one study tests the effect of adding different levels of antioxidant (Mottram et al., 1975). Thus data on the effect of adding different levels of ascorbate/ascorbic acid/erythorbic acid (i.e. varies forms of vitamin C) on the NA formation is needed in order to provide advice on the levels to be added during production and preferably regarding both VNA and NVNA.

The different forms of vitamin C are polar antioxidants and because both oxygen and nitrogen oxide produced by reduction of nitrite are more soluble in lipid (Combet et al., 2007) it has been suggested that the levels of nitrosating species produced in the lipid phase can be higher than in the aqueous lean phase of the meat. Nitrosating species liberated from the lipid phase have been suggested as the reason for the increase in NPYR during frying of bacon (Sen et al., 1976). Thus a combination of a polar (e.g. eryth-

orbic acid) and a non-polar antioxidant (e.g. ascorbyl palmitate) might inhibit the formation of NA more efficiently than one antioxidant. Polyphosphates are often used additives in meat processing because they increase the water holding capacity of the meat as well as stabilise the emulsion created in e.g. sausages (Bianchi, 1971). When the lipid and the lean phase are emulsified it might facilitate exchange of molecules between the two phases and thereby also exchange of nitrosating species and antioxidants.

Black pepper is used in the preparation of many types of sausages, including cooked sausages and salamis, and NPIP is often detected in these types of products (De Mey et al., 2014). Black pepper contains piperidine and *N*-nitrosopiperidine (Mey et al., 2014; Tricker, Pfundstein, Theobald, Preussmann, & Spiegelhalter, 1991); however, it has to our knowledge not been confirmed by controlled studies that the addition of black pepper to meat products results in occurrence of NPIP. NDMA, NPIP and NPYR may be produced when spices are mixed with nitrite, thus spices may be a source of NA precursors (Sen, Donaldson, Charbonneau, & Miles, 1974).

Haem has been suggested to play an essential role in the endogenous formation of NA following consumption of red and processed meat (Lunn et al., 2007; Pierre et al., 2013; Santarelli et al., 2010) and haem (Cross, Pollock, & Bingham, 2003) are associated with increased endogenous formation of nitroso compounds in both rodents and humans. Thus haem may play a role in the endogenous formation of nitroso compounds in general and therefore of NA as well. The haem iron may be released from myoglobin or haem as it passes through the digestive tract and it can therefore not be ruled out whether free iron plays a role in the nitroso compound formation. Free iron induces lipid oxidation processes in meat which is inhibited by the presence of antioxidants as is the NA formation, thus there may be some link between lipid oxidation processes and NA formation. The role of haem/myoglobin and free iron in NA formation in meat has to our knowledge not been studied.

To elucidate some of the areas mentioned above for which sufficient data is lacking we set up the following aims for the present study: (1) to examine the effect of the ingoing amount of nitrite on the amount of NA formed during the production of a cooked sausages; (2) to examine whether the formation of NA in the nitrite cured cooked sausages is affected by the presence of a water soluble (erythorbic acid) and a fat soluble antioxidant (ascorbyl palmitate), fat content, tripolyphosphate or black pepper; (3) to examine which concentrations of the two mentioned antioxidants that resulted in the most efficient inhibition of the formation of NA and if a combination of the two provides a higher degree of protection against NA formation than just one of them; (4) to examine whether haem or free iron play a role in the formation of NA in cooked sausages as has been suggested for the endogenous NA formation. In order to examine the effect of each individual factor, possible interactions between them and to work with a realistic number of experimental setups, factorial designs were employed.

## 2. Material and methods

### 2.1. Chemicals

Acetonitrile (extraction solvent) was of HPLC grade (Rathburn Chemicals Ltd., Walkerburn, Scotland). Formic acid (purity 98–100% for analysis), pipecolic acid (purity 98%), and the methanol for LC eluent (purity  $\geq 99.9\%$ , Fluka-Analytical) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Erythorbic acid, ascorbyl palmitate, tripolyphosphate, iron(III)sulphate hydrate and myoglobin from equine heart were also purchased from Sigma-Aldrich Co. The pure standards of *N*-nitrososarcosine

(NSAR), *N*-nitrosohydroxyproline (NHPR), *N*-nitrosodibenzylamine (NDBzA), *N*-nitrosoproline (NPRO), *N*-nitrosomethylaniline (NMA), *N*-nitroso-2-methyl-thiazolidine 4-carboxylic acid (NMTCA) and *N*-nitroso-thiazolidine-4-carboxylic acid (NTCA) were purchased from Toronto Research Chemicals (Toronto, Canada), whereas the standards *N*-nitrosodiethylamine (NDEA), *N*-nitrosodipropylamine (NDPA), *N*-nitrosomorpholine (NMOR) and *N*-nitrosodimethylamine (NDMA) were purchased from Sigma–Aldrich Co. *N*-nitrosomethylethylamine (NMEA), *N*-nitrosopyrrolidine (NPYR), *N*-nitrosodibutylamine (NDBA), *N*-nitrosopiperidine (NPIP) were purchased from Dr. Ehrenstorfer (Ausborg, Germany). The internal standards *N*-nitrosodimethylamine- $d_6$  (NDMA- $d_6$ ) and *N*-nitrosopyrrolidine- $d_8$  (NPYR- $d_8$ ) were purchased from Sigma–Aldrich Co. and CDN Isotopes (Quebec, Canada), respectively. The purity of all of the NA standards was  $\geq 98\%$  except for NMA which was of 95% purity. Sodium nitrite (Fluka-Analytical) was purchased from Sigma–Aldrich Co.

## 2.2. Cooked pork sausages (representative meat product)

Cooked pork sausages were chosen as representative meat products, because sausages account for a major part of the total consumption of processed meat products by the Danish, as well as other European populations (Linseisen et al., 2002). By choosing a minced meat product the ingredients are also more evenly distributed and any relevant equilibria are reached faster.

Trimmed fresh pork loin with a fat content of approximately 12% ([www.foodcomp.dk](http://www.foodcomp.dk)) was minced (Kenwood, MG470, Elgiganten, Glostrup Denmark) and all ingredients common for all samples in the setup were added during mixing (Bear Varimixer, AR5A, A/S Wodschow & Co., Broendby, Denmark). The sausage meat was prepared from tap water (26%), minced meat (67%), potato flour (4%), ground black pepper (*Piper nigrum*, Santa Maria A/S, Broendby, Denmark) (0.125 or 0.5%), sodium chloride (2%), paprika (0.5%), nitrite (0–350 mg kg<sup>-1</sup> depending on the setup). Aliquots of the thoroughly mixed sausage meat were transferred to a mini chopper (Phillips hand blender with chopper, HR1372, Punkt1, Roedovre, Denmark) and further mixed with the ingredients/factors to be tested and chopped until they were evenly distributed. The sausage meat that needed no further additions was chopped in the same way. Then the meat was filled into sheep casings using either a sausage stuffer for larger portions or a single use plastic piping bag equipped with a plastic stuffing horn for smaller portions. The sausages were hung on an oven grate and dried for 50 min at 70 °C in an oven for the larger portions (UNOX, XVC705, Vigodrzere-Podova, Italy) or a drying cabinet for smaller portions (Memmert drying cabinet, U40, Schwabach, Germany).

All sausages, in all four setups, were stored at –60 °C after the end of preparation and any other storage/treatments and until analysis.

## 2.3. Experimental design and statistical analysis

Four experimental setups were performed using cooked pork sausages as a model to meet the aims specified for the present study i.e. (1) A setup where the NA levels in cooked sausages prepared with six different levels of nitrite was determined. (2) A five factor 2-level factorial experiment to study the role of erythorbic acid, ascorbyl palmitate, fat content, tripolyphosphate and black pepper on the NA formation. (3) A full central composite experiment to study the effect of thirteen different combinations of five levels of erythorbic acid and five levels of ascorbyl palmitate on the NA formation. (4) To study the role of haem and iron in the NA formation a four factor 2-level factorial experiment was carried out including also erythorbic acid and calcium as factors. Whether there was a significant difference in the NA levels in sausages pre-

pared with the high or the low level of each of the factors in the two 2-level factorial experiments was tested by Student's *t*-test at 95% confidence levels. The statistical software Minitab (version 16) was used for setting up the design and for the analysis of the results.

## 2.4. Role of the ingoing amount of nitrite, drying and storage time (first setup)

The role of different levels of the in going amount of nitrite on the NA formation in sausages, the influence of storage time after preparation and the effect of frying the sausages was studied. First sausages were prepared in accordance with the recipe described above only varying the amount of in going nitrite. Six levels of nitrite was employed i.e. 0, 60, 100, 150, 250 and 350 mg kg<sup>-1</sup>. Twenty-four sausages, of approximately 25–35 g each, were prepared for each level of nitrite, i.e. a total of 144 sausages. The 24 sausages prepared for each of the six nitrite levels were divided into four sub-groups. Sub-group 1 was packed immediately after preparation ( $t_0$ ) and put into the freezer after approximately 4 h. Sub-group 2 was packed immediately after the drying process ( $t_1$ ) and frozen after about 2 h. Sub-group 3 and 4 were packed immediately after the drying process and stored at 5 °C for 24 h ( $t_2$ ) and then frozen. Sub-group 4 was used for studying the effect of pan frying. These sausages were fried for 10 min one group at a time using the same frying pan. During these 10 min the centrum temperature of the sausage reached 100 °C. The weight loss was registered allowing for calculation of the NA content per kg<sup>-1</sup> of the sausages not fried.

## 2.5. Role of erythorbic acid, ascorbyl palmitate, fat content, black pepper and tripolyphosphate (second setup)

This second setup, a 2-level fractional factorial design (resolution V), was set up to study the effect of erythorbic acid (250 or 1000 mg kg<sup>-1</sup>), ascorbyl palmitate (0 or 250 mg kg<sup>-1</sup>), increased fat content (12 or 25%), black pepper (1.25 or 5 g kg<sup>-1</sup>) and tripolyphosphate (0 or 5 g kg<sup>-1</sup>) on the formation of NA in cooked sausages prepared with 150 mg kg<sup>-1</sup> sodium nitrite. The design included the preparation of sausages with 16 different combinations of the five factors. A minimum of six sausages, each of about 15 g, were prepared for each of the 16 preparations. The sausages were packed in sealed plastic bags with minimum three in each. One bag of each of the 16 preparations was stored either for 24 h or 5 days at 5 °C before freezing.

## 2.6. Mitigation by erythorbic acid and/or ascorbyl palmitate (third setup)

The third setup, a full central composite experimental design, included 13 combinations of five different concentrations of erythorbic acid (396, 500, 750, 1000 and 1104 mg kg<sup>-1</sup>) and ascorbyl palmitate (26, 150, 450, 750 and 874 mg kg<sup>-1</sup>) in cooked sausages prepared with 150 mg kg<sup>-1</sup> sodium nitrite. These 13 combinations included four samples representing a “cube” portion, two axial or “star” points, a center point and four replicates. Four sausages, each of approximately 15 g, were prepared for each of the 13 combination of the two antioxidants.

## 2.7. Role of haem and iron (fourth setup)

The role of haem iron, in the form of myoglobin from equine heart, and free iron, in the form of iron(III)sulphate hydrate, in the formation of NA in cooked sausages prepared with 150 mg kg<sup>-1</sup> sodium nitrite was studied. Calcium, in the form of calcium carbonate, and erythorbic acid was also included as a factor in this fac-

torial design because these two factors may counteract a possible effect of haem and iron, respectively. By including erythorbic acid in this setup it was also possible to test the effect of this factor again. The full 2-level factorial design setup required preparation of sausages from sausage meat prepared with 16 different combinations of the four factors, i.e. added myoglobin (0 or 1.5 g kg<sup>-1</sup>), iron(III)sulphate hydrate (0 or 36 mg kg<sup>-1</sup>), erythorbic acid (0 or 1000 mg kg<sup>-1</sup>) and/or calcium carbonate (0 or 6 g kg<sup>-1</sup>). Four sausages, of approximately 15 g each, were prepared for each of the 16 preparations.

## 2.8. N-nitrosamine analysis

The contents of eight VNA and five NVNA in the samples were determined according to a method recently developed and validated at our laboratory (Herrmann, Duedahl-Olesen, & Granby, 2014). In the following the method will only be described in brief.

## 2.9. Extraction method

2.5 g of homogenised sample with internal standard (ISTD) added (NPYR-*d*<sub>8</sub> and NDMA-*d*<sub>6</sub>) was extracted with 7.5 ml 1% formic acid in acetonitrile. After centrifugation the supernatant was removed and frozen. The thawed extract was centrifuged (4500g). 5 ml of the acetonitrile phase was evaporated under a stream of nitrogen to a volume of ~0.25 ml and then adjusted to 1.0 ml with Milli-Q water. After diluting 1:1 with Milli-Q water the extract was filtered and analysed.

## 2.10. LC-MS/MS method

The final extracts were analysed by LC(APCI/ESI)-MS/MS as described in Herrmann et al. (2014). The analytical platform consisted of an Agilent 1200 Series HPLC (Agilent Technologies, Santa Clara, CA, US) coupled with an Agilent 6460 Series Triple Quadrupole

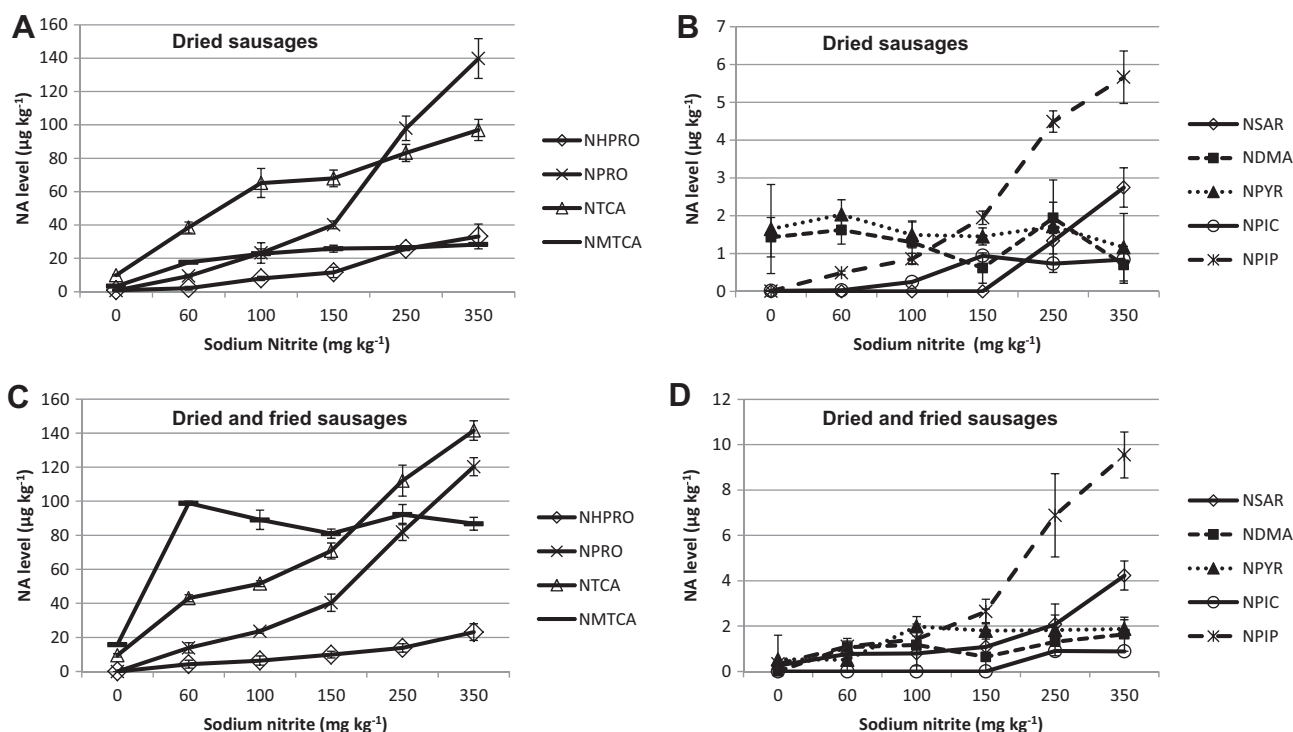
(Agilent Technologies) equipped with either an APCI or a Jet Stream ESI source. The analytical column was a Poroshell PhenylHexyl column 150 × 2.1 mm, 3 µm column (Agilent Technologies). A mobile phase gradient programme was applied, using 0.1% formic acid in water and methanol, respectively. The injection volume was 3.5 µl.

Quantitative and qualitative analysis were performed by external calibration (0.334 to 1000 ng ml<sup>-1</sup>) and compared with the retention times and quantifier ion/qualifier ion ratios obtained by analysing NA standard solution and/or spiked QC samples (Herrmann et al., 2014).

## 3. Results and discussion

### 3.1. Role of the ingoing amount of nitrite, drying and time (first setup)

By increasing the ingoing amount of nitrite (0, 60, 100, 150, 250, 350 mg kg<sup>-1</sup>) the levels of NHPRO, NPRO, NTCA, NMTCA (Fig. 1A), NSAR and NPIP (Fig. 1C) increased in the sausages. A steep increase in the level of NMTCA was observed by adding 60 mg kg<sup>-1</sup>. Higher levels of nitrite only increased the NMTCA levels slightly, indicating that other factors than nitrite is the limiting factor for the formation of NMTCA. In sausages prepared with 150 mg kg<sup>-1</sup> nitrite, which is the amount of nitrite allowed to be added to sausages for the common European market ([https://webgate.ec.europa.eu/sanco\\_foods](https://webgate.ec.europa.eu/sanco_foods)), NPIP (Fig. 1C), NHPRO, NPRO, NTCA and NMTCA (Fig. 1A) were found in levels of approximately 2, 10, 40, 70 and 25 µg kg<sup>-1</sup>, respectively. NSAR was at LOD if more than 150 mg kg<sup>-1</sup> nitrite was added, and by further increasing the nitrite level a clear increase in the NSAR level was found (Fig. 1C). The levels of NDMA and NPYR were relatively unaffected by the increase in added nitrite. The levels of NDMA and NPYR remained at or below 2 µg kg<sup>-1</sup>, which is at the limit of quantification (LOQ) for the method applied (Herrmann et al., 2014). Increasing the level of nitrite was also found by others to have a limited effect on the level of NDMA (Drabik-Markiewicz et al., 2011).



**Fig. 1.** Level of NA in sausages prepared with 0, 60, 200, 150, 250 or 350 mg kg<sup>-1</sup> sodium nitrite. The NVNA occurred at high levels (A and B), whereas the VNA occurred at lower levels (C and D) in sausages analysed after drying and 24 h of storage at 5 °C (A and C) and sausages which were also further prepared by pan frying for 10 min until a core temperature of 100 °C (B and D). The levels found in the fried sausages have been corrected for weight loss during frying, i.e. presented in µg kg<sup>-1</sup> sausage before frying.



If the sausages were further prepared by pan frying (Fig. 1B and C) the levels of NSAR, NPIP (Fig. 1D), NTCA and NMTCA (Fig. 1B) increased by up to about 2, 2, 1.5 and 4 times, respectively. For NTCA the difference in the content between the not fried (Fig. 1A) and the fried sausages (Fig. 1B) increased with increasing amount of ingoing nitrite. This resulted in a more linear correlation between added nitrite and NTCA level and with a steeper slope than found for the not fried sausages. For these fried sausages a slightly higher level of NDMA and NPYR were indicated for the sausages prepared with 60 or 100 mg kg<sup>-1</sup> nitrite than in those prepared without nitrite (Fig. 1D). In the sausages prepared with 150 mg kg<sup>-1</sup> nitrite the levels of NPIP (Fig. 1D), NHPRO, NPRO, NTCA and NMTCA (Fig. 1B) amounted to 2.6, 10, 40, 70 and 80 µg kg<sup>-1</sup>, thus frying induced an increase in the NPIP (2.6 µg kg<sup>-1</sup>) and the NMTCA (80 µg kg<sup>-1</sup>) levels.

The levels of NDMA and NPYR remained at or below 2 µg kg<sup>-1</sup> also in the fried sausages. NTCA levels of up to 140 µg kg<sup>-1</sup> were detected in the sausages after frying. Thus relatively high levels of NTCA may be produced when sausages are fried until a center temperature of 100 °C. The high levels of NTCA reported for smoked meat products (Massey, Key, Jones, & Logan, 1991) may be at least partly attributed to the heat treatment (60–80 °C) which is also performed during traditional hot smoke processing (Fellows, 2009). However if heated to a temperature of 250 °C for approximately 10 min. studies performed at our laboratory have shown that the levels of both NTCA and NMTCA decrease (Herrmann, Duedahl-Olesen, & Granby, 2015). This decrease may be caused by heat induced decarboxylation of NTCA and NMTCA to NTHz and NMTHz, respectively. Though according to Mandagere, Gray, Ikins, Booren, and Pearson (1987) the levels of NTHz also decrease during frying of bacon.

Only slight differences in the NA levels were observed between sausages frozen immediately after preparation (without drying process) ( $t_0$ ), immediately after drying ( $t_1$ ) and sausages frozen after drying and 24 h of storage at 5 °C ( $t_2$ ) (Fig. 2). Though, the levels of NTCA were affected by the drying process, i.e. increased from approximately 10 µg kg<sup>-1</sup> ( $t_0$ ) to 55 and 85 µg kg<sup>-1</sup> ( $t_1$ ) in sausages prepared with 150 and 350 mg nitrite kg<sup>-1</sup>. Storage for 24 h at 5 °C ( $t_2$ ) did not further affect the levels of NTCA.

The present study showed that when the ingoing amount of nitrite increases, the levels of most NA also increase. Only for NDMA and NPYR this relationships was not found. In general however the results for NDMA and NPYR in the present study can only be indicative because the levels of these two NA were at the LOQ level and therefore associated with higher uncertainties.

### 3.2. Role of erythorbic acid, ascorbyl palmitate, fat content, black pepper and tripolyphosphate (second setup)

Fig. 3A1–E1 shows the main effects, i.e. the effect of the individual factors, on the NA levels in sausages. Of the five factors studied in the factorial design it was found that the two antioxidants, erythorbic acid and ascorbyl palmitate, had the highest impact on the levels of NA (Fig. 3A1, B1, D1 and E1). In general the increasing the level of or adding antioxidants lowered the levels of NAs in the sausages. The levels of NSAR, NDMA and NPYR were at the limit of determination (LOD) or LOQ and the observed effects are therefore associated with great uncertainty. No figures have therefore been generated for these three NA since the observed effects can only be indicative. Mottram, Patterson, Edwards, and Gough (1977) showed however that NDMA formation is inhibited by ascorbate. They produced an NDMA level of 100 µg kg<sup>-1</sup> pork meat by fortifying meat with dimethylamine (100 ppm) and curing it in brine with 1000 ppm NaNO<sub>2</sub>. By also adding 2000 ppm of ascorbate to the brine the level of NDMA decreased to <1 µg kg<sup>-1</sup> (Mottram et al., 1975).

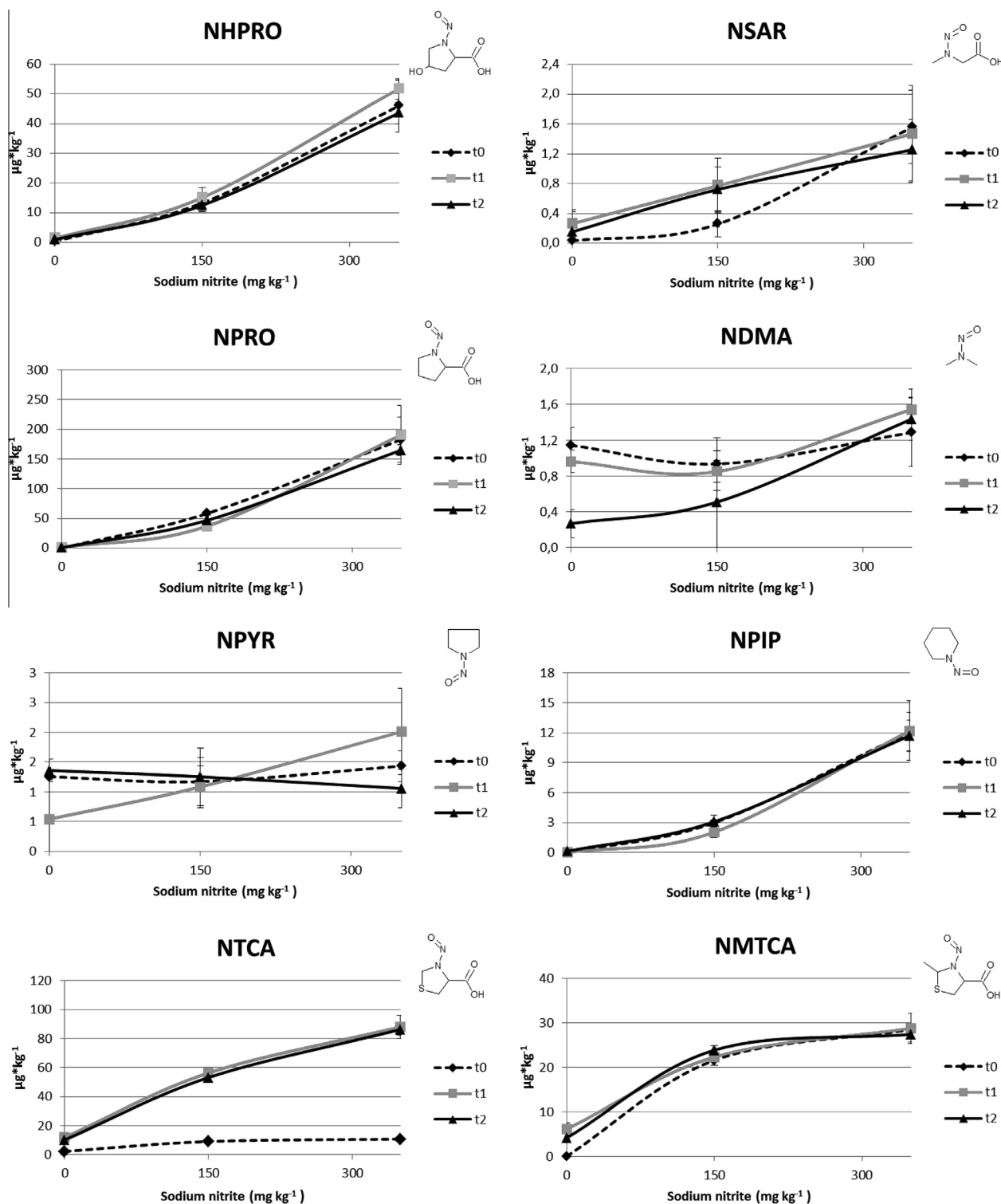
In the present setup the levels of NPIP (Fig. 3C1) were not reduced by increasing the level of erythorbic acid or adding ascorbyl palmitate. The levels of NSAR and NDMA also did not seem to be affected by the presence of erythorbic acid and ascorbyl palmitate. For NPIP the low level of erythorbic acid of 250 mg kg<sup>-1</sup> though seems to provide the full inhibitory effect. This is indicated by the approximate 60% reduction in the NPIP levels observed for sausages prepared with 1000 mg kg<sup>-1</sup> erythorbic acid compared to no erythorbic acid in the setup four (Fig. 5C1).

No significant effects were induced by increasing the fat content from 12% to 25%, though a slight increase in the levels of NDMA and NPYR, as well as a decrease in the levels of NSAR and NMTCA, was indicated. The slightly higher levels of NDMA and NPYR are in agreement with the results of e.g. Mottram et al. (1977) who found that NDMA and NPYR formation was primarily occurring in the lipid phase of bacon. Several mechanism for this preferential formation in the lipid phase has been presented; higher temperature during frying than in the lean part with a higher water content, a different chemical environment favouring nitrosation (Mottram et al., 1977) which could give a higher solubility of both nitrogen oxide (NO) and oxygen in the lipid phase (Liu, Miller, Joshi, Thomas, & Lancaster, 1998) resulting in higher levels of nitrifying species as e.g. N<sub>2</sub>O<sub>2</sub>.

The level of NPIP increased from approximately 0.1 to 0.4 µg kg<sup>-1</sup> when increasing the amount of black pepper from 1.25 to 5.0 g kg<sup>-1</sup> sausage meat (Fig. 3C1). Though, the effect was not significant. However if applying the same analysis and data treatment to the same type of sausages stored for additionally four days at 5 °C before freezing, the level of NPIP was significantly higher in the sausages with the high amount of black pepper than in the sausages with the low amount (data not shown). Besides the higher level of NPIP only minor differences in the NA levels were observed for the sausages stored for 24 h and those stored for 5 days at 5 °C. When preparing the sausages with 5.0 g of black pepper per kg sausage meat and without any antioxidants the levels of NPIP were in the order of 2.0 (setup one) to 2.7 µg kg<sup>-1</sup> (setup four). The present study supports, that NPIP in processed meat products originates or partly originates from the use of black pepper. Yurchenko and Mölder (2007) also suggested that black pepper may be the main source of NPIP.

The level of NMTCA was also significantly increased by an increase in the amount of black pepper (Fig. 3E1). A pepper induced increase was also indicated for NTCA (Fig. 3D1). NTCA (Ratner & Clarke, 1937) and NMTCA are formed by the condensation of formaldehyde or acetaldehyde with cysteine followed by nitrosation. The formation of these two NA may therefore be limited by the availability of the aldehydes, cysteine or the actual precursors, i.e. thiazolidine 4-carboxylic acid (TCA) and 2-methylthiazolidine 4-carboxylic acid (MTCA).

In meat the presence of the amino acid cysteine ought not to be a limiting factor for the formation of NTCA or NMTCA. TCA occurs in plants at varying levels (Suvachittanont, Kurashima, Esumi, & Tsuda, 1996) with pepper being a potential source of TCA (Fig. 6). Formaldehyde is ubiquitous in the environment and exists at low levels in most living organisms as a metabolic intermediate; however, black pepper also contains substances (e.g. piperine) which can liberate formaldehyde. Larger amount of formaldehyde is liberated during combustion processes and therefore also produced during wood smoking. High levels of NTCA occur in smoked meat products. The formation of NTCA therefore seems to be limited by the availability of formaldehyde. Formation of NMTCA seems to be less related to the smoking process (Herrmann et al., 2015; Massey et al., 1991) and dependent on other constituent(s). As mentioned earlier we performed some preliminary tests on a simpler meat system using minced pork meat, to which only water, nitrite and sodium chloride were added. In this simple meat



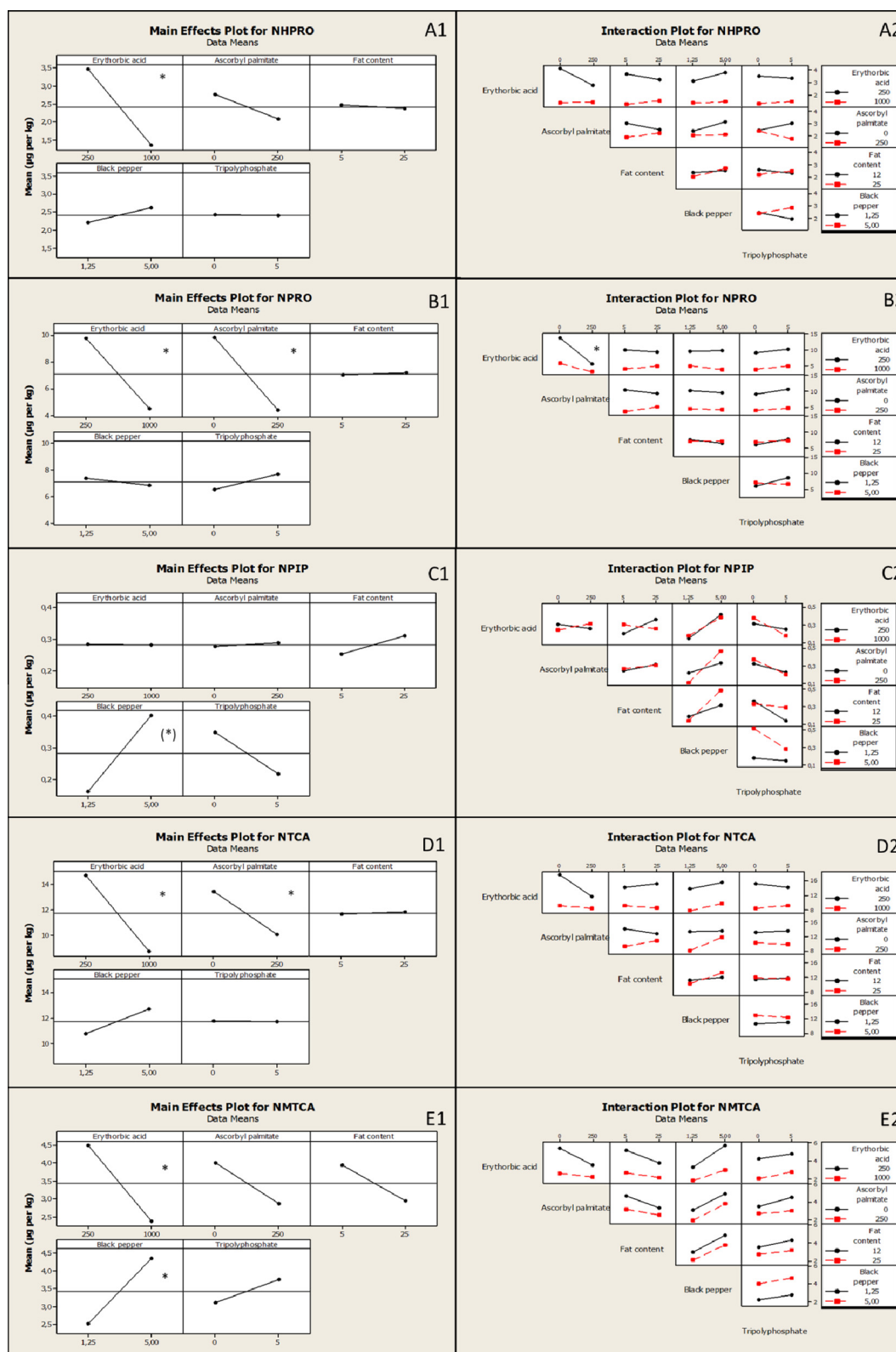
**Fig. 2.** N-nitrosamine levels in sausages prepared with sodium nitrite (0, 150 or 350  $\text{mg}\cdot\text{kg}^{-1}$ ) without drying ( $t_0$ ) with drying for 50 min at 70 °C ( $t_1$ ) and further storage for 24 h at 5 °C ( $t_2$ ). All samples were analysed in triplicate and the error bars show the standard deviation on the results.

system we found that the formation of both NTCA and NMTCA was only limited by nitrite, because saturation curves were observed with increasing incoming amount of nitrite (data not shown).

The addition of tripolyphosphate resulted in no significant main effects (Fig. 3A1–E1).

In Fig. 3A2–E2 are the observed interactions presented as interaction plots. If the lines in the interaction plots are parallel it

indicates no interaction between the two factors in question (indicated below and in the right side of the figure). Only one significant interaction was observed in this setup. If the level of erythorbic acid was high then the effect of also adding ascorbyl palmitate on the NPRO level (Fig. 3B2) was very limited, whereas if the level of erythorbic acid was low adding ascorbyl palmitate did provide further inhibition. This interaction was also indicated for the other

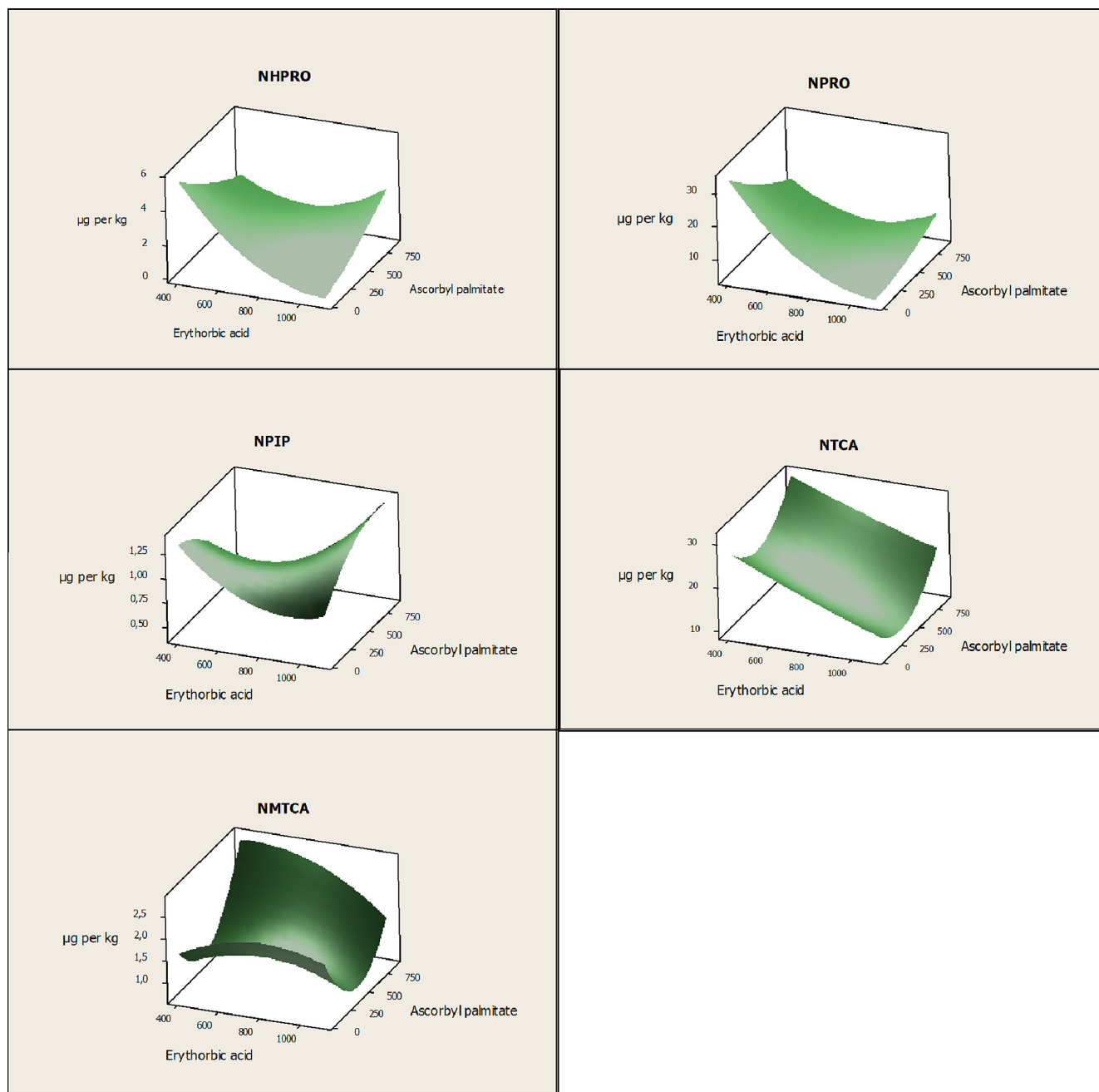


**Fig. 3.** Main effects (A1–E1) and interactions (A2–E2) of erythorbic acid (250 or 1000 mg kg<sup>-1</sup>), ascorbyl palmitate (0 or 250 mg kg<sup>-1</sup>), fat content (~12 or ~25%) and black pepper (1.25 or 5 g kg<sup>-1</sup>) on the N-nitrosamine levels in sausages prepared with 150 mg kg<sup>-1</sup> sodium nitrite and stored for 24 h at 5 °C. Data analysis was performed using Minitab (version 6). \*The effect or interaction is statistical significant (95% confidence level). \*The effect was significant in sausages stored for five days (unpublished data) instead of 24 h at 5 °C.

NA. From the interaction plots it also appears that the distance between the two lines are generally greatest for erythorbic acid which very nicely illustrates that of the tested factors erythorbic acid exhibits the largest effect on the NA levels.

Based on the result of this second setup we concluded that black pepper increases the levels of at least two NA of which one is known to be carcinogenic. Besides the ingoing amount of nitrite, erythorbic acid is the factor with the highest impact on the NA





**Fig. 4.** Response surface plots for the effect of erythorbic acid (396, 500, 750, 1000 and 1104 mg kg<sup>-1</sup>) and ascorbyl palmitate (26, 150, 450, 750 and 874 mg kg<sup>-1</sup>) on the NA levels in sausages prepared with sodium nitrite (150 mg kg<sup>-1</sup>).

levels. Ascorbyl palmitate may contribute to the inhibition of NA and it was therefore chosen to further examine the effect of combining the two antioxidants at different levels (third setup).

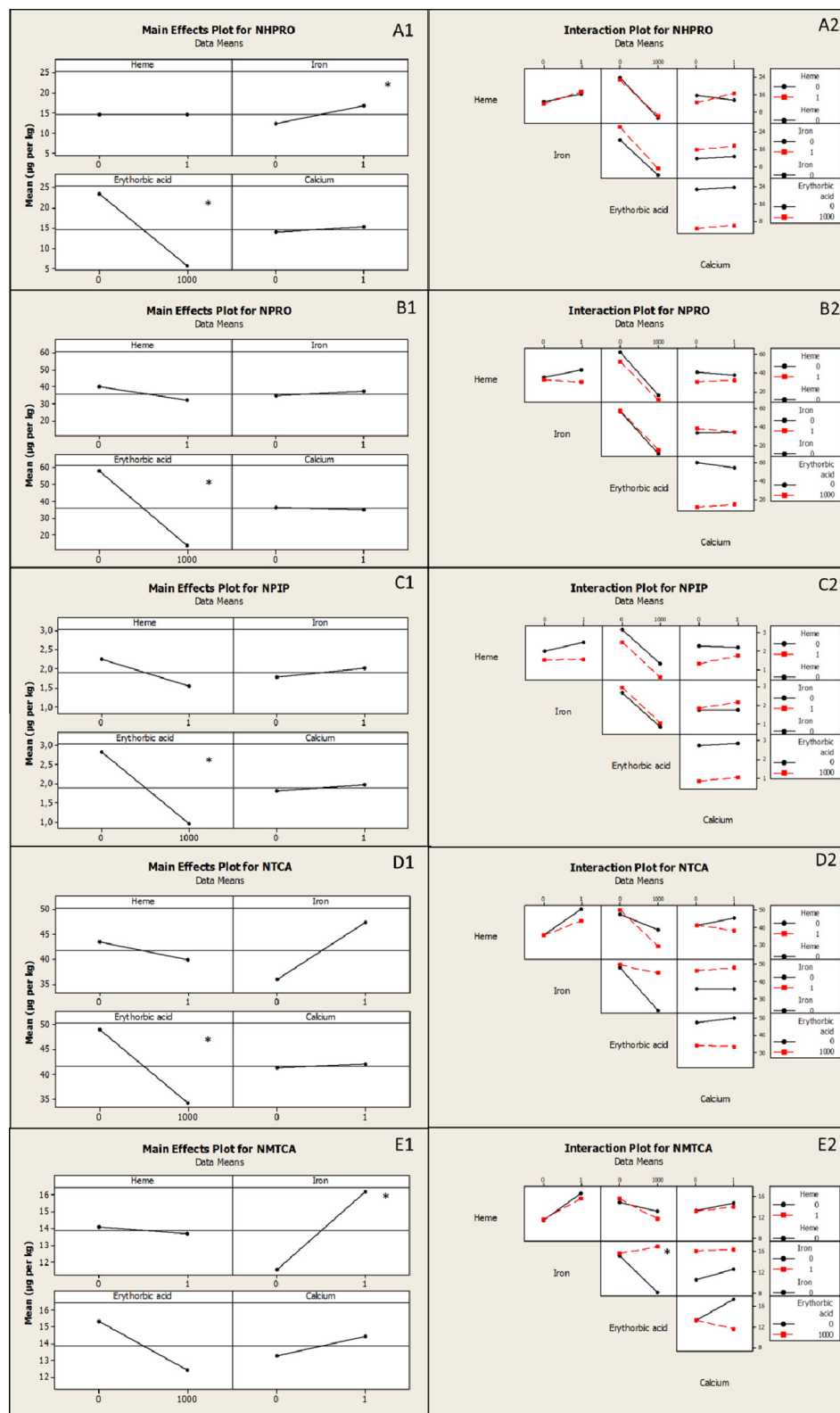
### 3.3. Mitigation by erythorbic acid and/or ascorbyl palmitate (third setup)

The results of this third setup are illustrated as surface plots (Fig. 4). As can be seen from these surface plots the levels of NHPRO, NPRO, NPIP and NTCA decrease with increasing amount of erythorbic acid (396, 500, 750, 1000 and 1104 mg kg<sup>-1</sup>). The levels of NDMA, NSAR and NPYR were also in this setup up low (~1 µg kg<sup>-1</sup>) in all thirteen samples and the results obtained for these NA are therefore associated with great uncertainty and surface plots have therefore not been generated for these two NA.

The present study demonstrates that the levels of NA in sausages may be reduced by adding erythorbic acid and that the extent of the inhibition increases with increasing amount of erythorbic acid, at least up to the highest tested level of 1104 mg kg<sup>-1</sup>. The effect of concurrent presence of ascorbyl palmitate (26, 150, 450, 750 and 874 mg kg<sup>-1</sup>) however seems to have the opposite effect, i.e. to stimulate the formation of NA or to counter act the effect of erythorbic acid.

### 3.4. Role of haem and iron (fourth setup)

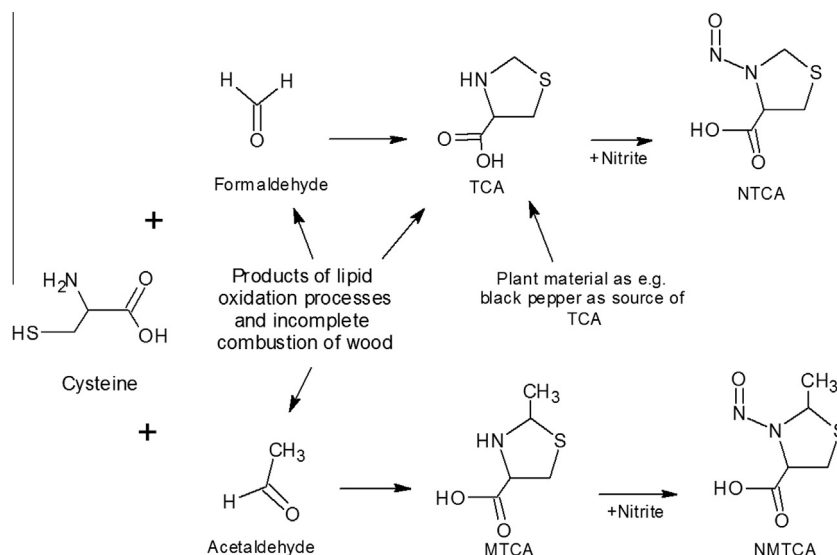
Haem has been suggested to play an essential role in the endogenous formation of NA linked with high consumption of red and processed meat (Bingham, Hughes, & Cross, 2002; Cross et al., 2003; Pierre et al., 2013). Lunn et al. (2007) also found that in an



**Fig. 5.** Main effects (A1–E1) and interactions (A2–E2) of heme as myoglobin (0 or 1.5 g kg<sup>-1</sup>), iron as iron(III)sulphate hydrate (0 or 36 mg kg<sup>-1</sup>), erythorbic acid (0 or 1000 mg kg<sup>-1</sup>) and calcium as calcium carbonate (0 or 6 g kg<sup>-1</sup>) on the N-nitrosamine levels in sausages prepared with 150 mg kg<sup>-1</sup> sodium nitrite and stored for 24 h at 5 °C. Data analysis was performed using Minitab (version 6). \*The effect or interaction is statistical significant (95% confidence level).

aqueous solution no nitrosation of morpholine occurred even at elevated levels of nitrite, however if haem iron was also added NMOR was produced (Lunn et al., 2007). Calcium, which can

chelate the iron in haem, was by Pierre et al. (2013) found to prevent a haem induced increase in endogenous formation of nitroso compounds in humans (Pierre et al., 2013). Iron plays an essential



**Fig. 6.** Formation of *N*-nitrosothiazolidine-4-carboxylic acid (NTCA) and *N*-nitroso-2-methyl-thiazolidine-4-carboxylic acid (NMTCA) from condensation of cysteine with formaldehyde or acetaldehyde with subsequent nitrosation of the formed thiazolidine-4-carboxylic acid (TCA) and 2-methyl-thiazolidine-4-carboxylic acid (MTCA) precursor. The aldehydes may originate from lipid oxidation processes or from wood smoke and/or TCA may originate from plant material added in the form of spices or other ingredients.

role in lipid peroxidation processes occurring in meat and antioxidants as ascorbate/erythorbic acid inhibit these unwanted processes (Igene, King, Pearson, & Gray, 1979; Ladikos & Lougovois, 1990).

The results of the study are presented in Fig. 5. The levels of NSAR, NDMA and NPYR were in all the tested combinations at or below the LOQ of the method. The observed effects on the levels of these three NA are therefore associated with high uncertainty and the results for these are not included in Fig. 5. The observations are however described as indicative results in the following.

No significant effects were observed by supplementing the sausage meat with haem (myoglobin) (Fig. 5A1–E1). However a slight reduction in the levels of NPIP (Fig. 5C1), NSAR and NPYR were indicated. This reduction may be the result of increased competition for the nitrosating species because more NO was bound to the added haem. Slight indications of calcium counteracting this inhibiting effect were observed for NHPRO (Fig. 5A2), NDMA and NPYR.

The levels of NHPRO and NMTCA were found to increase significantly by adding Fe (III) (Fig. 5A1 and E1). An increase was also indicated for NTCA (Fig. 5D1) and NPYR. For the remaining NA no effect was observed by adding Fe(III). Erythorbic acid was also in this setup found to reduce the NA levels (Fig. 5A1–E1) except for NDMA and NPYR. The reduction was found to be significant for NHPRO, NPRO, NPIP and NTCA. Interaction between iron and erythorbic acid was indicated for NTCA and NMTCA, though only significantly for NMTCA (Fig. 5E2). Addition of Fe(III) cancelled the otherwise inhibiting effect of erythorbic acid (Fig. 5D2 and E2). The fact that erythorbic acid does not result in a decrease in the levels of NTCA and NMTCA if the sausage meat has also been supplemented with Fe(III) may indicate that the degree of oxidative stress is too severe for the added amount of prooxidant to have an effect, or that the inhibitory effect of adding erythorbic acid is counteracted by an increasing level of Fe(II).

Thus the present study does not indicate that haem is a catalyst for the formation of NA in meat product as has been suggested for endogenous formation. It does however indicate that free iron may stimulate the formation of NA in meat and that the effect of adding antioxidants as erythorbic acid which normally reduces the levels of NA is diminished or prevented by the elevated iron level. This effect was especially clear for NTCA and NMTCA. The formation

of NTCA, NMTCA was also prevented to a lesser extent by just the presence of erythorbic acid than was NHPRO, NPRO and NPIP. The levels of these three NA were reduced by approximately 60–75% by the addition of the 1000 mg kg<sup>−1</sup> erythorbic acid.

The observed interaction between Fe and erythorbic acid may indicate that the formation of NTCA and NMTCA are linked to oxidative processes occurring in the meat. Oxidation of phosphor lipids actually results in the formation of many different aldehydes (Esterbauer, Schaur, & Zollner, 1991) including formaldehyde (Farmer & Mottram, 1990) and perhaps also acetaldehyde (Fig. 6). Lipid oxidation processes are promoted by heat and prolonged storage under aerobic conditions. Storage for 24 h of uncooked sausage meat at room temperature and aerobic conditions resulted in four times higher levels of NTCA (10 compared to 40 µg kg<sup>−1</sup>) and NMTCA (3 compared to 12 µg kg<sup>−1</sup>) than if the same samples were stored at 5 °C in a tight container. A four-fold higher level of NTCA and NMTCA by a temperature increase of 15 °C corresponds well to a general temperature coefficient by a factor of 2 for a 10 °C increase in temperature which has been found to apply to biological and chemical reactions in general. The higher levels produced in the sample stored at room temperature under aerobic may have resulted in more lipid oxidation. Smoke is though also a significant source of aldehydes (Ikins et al., 1988) why the highest levels of NTCA are found in smoked products (Herrmann et al., 2015; Sen, Baddoo, & Seaman, 1986). Several aldehydes may occur in the products but e.g. formaldehyde and acetaldehyde can upon reaction with cysteine from the meat and subsequent nitrosation produce NTCA and NMTCA, respectively (Ohshima & Bartsch, 1984). The saturation curves observed for the formation of NTCA and NMTCA in relation to added nitrite in the minced meat model, as described earlier (data not shown), may thus indicate that the amount of precursors was limited. This may be due to a low degree of lipid peroxidation and/or that ingredients added to the sausages, but not to the minced meat model, contain the relevant precursors (Fig. 6).

#### 4. Conclusion

From the present results it can be concluded that there is a positive correlation between the amount of ingoing nitrite and the lev-

els of the targeted NA formed in sausages prepared from minced pork meat. An exception was NDMA and NPYR of which the formation seemed unaffected by increasing amount of nitrite.

Among several factors which can easily be controlled during the production, erythorbic acid was the factor which affected the levels of most NA in nitrite preserved sausages. The levels of most NA were found to be inversely proportional with increasing amounts of erythorbic acid (396–1104 mg kg<sup>-1</sup>). By preparing the sausages with 1000 mg kg<sup>-1</sup> erythorbic acid the levels of the different NA were reduced by 20–75%. The formation of NPIP was found to be closely linked to the amount of black pepper added. Adding ascorbyl palmitate, a fat soluble antioxidant, provided no additional protection from NA formation than a high level of erythorbic acid (1000 mg kg<sup>-1</sup>) alone. Free iron and not haem stimulated the formation of some NA (NMTCa and NHPRo and maybe also NTCA) in sausages. Addition of free iron counteracted the inhibitory effect of erythorbic acid on the formation of NTCA and NMTCa.

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